

Dot/Icm Type IVB Secretion System Requirements for *Coxiella burnetii* Growth in Human Macrophages

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ABSTRACT Central to Q fever pathogenesis is replication of the causative agent, *Coxiella burnetii*, within a phagolysosome-like parasitophorous vacuole (PV) in mononuclear phagocytes. *C. burnetii* modulates PV biogenesis and other host cell functions, such as apoptotic signaling, presumably via the activity of proteins delivered to the host cytosol by a Dot/Icm type IVB secretion system (T4BSS). In this study, we utilized a *C. burnetii* strain carrying IcmD inactivated by the *Himar1* transposon to investigate the requirements for Dot/Icm function in *C. burnetii* parasitism of human THP-1 macrophage-like cells. The *icmD::Tn* mutant failed to secrete characterized T4BSS substrates, a defect that correlated with deficient replication, PV development, and apoptosis protection. Restoration of type IVB secretion and intracellular growth of the *icmD::Tn* mutant required complementation with *icmD*, *-J*, and *-B*, indicating a polar effect of the transposon insertion on downstream *dot/icm* genes. Induction of *icmDJB* expression at 1 day postinfection resulted in *C. burnetii* replication and PV generation. Collectively, these data prove that T4BSS function is required for productive infection of human macrophages by *C. burnetii*. However, illustrating the metabolic flexibility of *C. burnetii*, the *icmD::Tn* mutant could replicate intracellularly when sequestered in a PV generated by wild-type bacteria, where Dot/Icm function is provided in *trans*, and within a phenotypically similar PV generated by the protozoan parasite *Leishmania amazonensis*, where host cells are devoid of Dot/Icm T4BSS effector proteins.

IMPORTANCE *Coxiella burnetii*, the cause of human Q fever, is the only bacterial pathogen known to replicate in a vacuole resembling a phagolysosome. The organism manipulates host macrophages to promote the biogenesis of a vacuolar compartment permissive for growth. By analogy to the well-established cellular microbiology of *Legionella pneumophila*, the Dot/Icm type IVB secretion system of *C. burnetii* is implicated as a critical virulence factor in host cell modification that delivers proteins with effector functions directly into the host cell cytosol. Using new genetic tools, we verify that Dot/Icm function is essential for productive infection of human macrophages by *C. burnetii*. Interestingly, despite the production of homologous secretion systems, *L. pneumophila* and *C. burnetii* have strikingly different temporal requirements for Dot/Icm function during their respective infectious cycles.

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The zoonotic disease agent *Coxiella burnetii* is a highly infectious Gram-negative bacterium that causes human Q fever (1). Aerosol-transmitted *C. burnetii* has a tropism for mononuclear phagocytes such as alveolar macrophages (2, 3). Consequently, infection of cultured primary or immortalized human monocytes/macrophages is considered the most physiologically relevant *in vitro* model of *C. burnetii*-host cell interactions (4). Regardless of the host cell type, *C. burnetii* replicates within a membrane-bound compartment or “parasitophorous vacuole” (PV). The *C. burnetii* PV is unique among vacuoles occupied by bacterial pathogens in maturing through the endolysosomal pathway to ultimately acquire characteristics of a phagolysosome (4, 5). Here, the pathogen resists lysosomal hydrolases while exploiting the acidic pH for metabolic activation (6).

The notion of the *C. burnetii* PV as a large phagolysosome is oversimplified. Indeed, the PV also has interactions with autophagic and secretory pathways that promote *C. burnetii* replication (7–9). A mature PV filled with *C. burnetii* can occupy nearly the entire host cell cytoplasm (10); thus, extensive recruitment of membrane from other host vesicular compartments must occur during the PV expansion phase. Cumulative evidence indicates that *C. burnetii* actively mediates PV fusogenicity/maintenance (11) and other host cell processes, such as autophagy (9) and apoptosis (12–15), that benefit pathogen growth. Inhibition of apoptosis is considered a pathogenic strategy that accommodates the slow growth rate of *C. burnetii* (15), while fusion with autophagosomes may provide critical nutrients (9).

Type IVA and IVB secretion systems are critical bacterial viru-

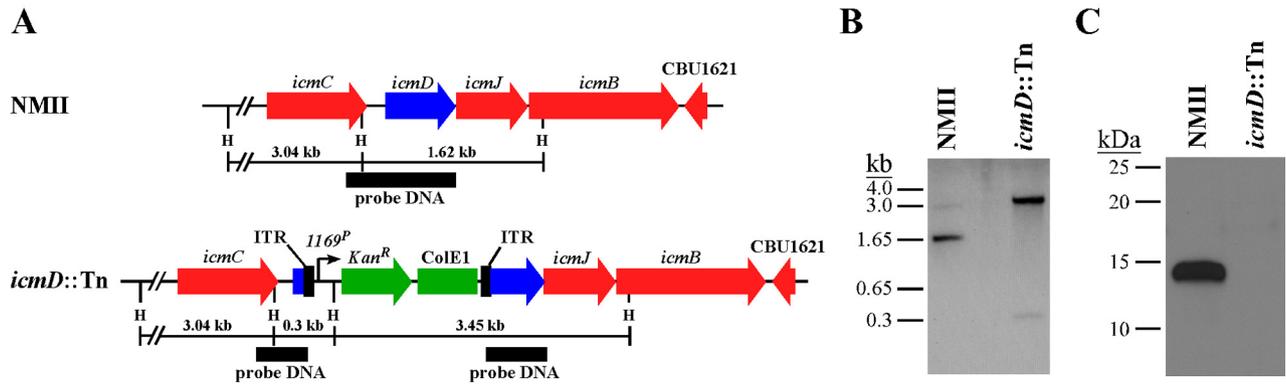


FIG 1 A *Himar1* transposon insertion inactivates *C. burnetii* *icmD*. (A) Schematic showing the location of a *Himar1* transposon insertion in *icmD*. The transposon inserted at base 75 of the 414-bp gene. ITR, inverted terminal repeat; H, HindIII site. (B) Southern blot assay of HindIII-digested genomic DNA from NMII and the *icmD::Tn* mutant hybridized with a probe specific to *icmD* (black bar in panel A). A 1.62-kb *icmD*-containing HindIII fragment was disrupted in the *icmD* mutant. (C) Immunoblotting of lysates from NMII and the *icmD::Tn* mutant grown in ACCM-2 for 6 days. IcmD-specific antiserum detected the 14.3-kDa IcmD protein in lysates from NMII but not in those of the *icmD::Tn* mutant.

lence factors that deliver effector proteins directly to the host cytosol (16). Circumstantial evidence suggests that proteins translocated by a Dot/Icm type IVB secretion system (T4BSS) are critical for successful parasitism of macrophages by *C. burnetii* (17). This model is based on the well-established role of the homologous Dot/Icm T4BSS of *Legionella pneumophila* in subverting host cell processes, such as organelle trafficking, to generate a permissive intracellular growth environment (18). The *C. burnetii* genome contains 23 homologues of the 26 *Legionella dot/icm* genes (19), and 62 *C. burnetii* Dot/Icm substrates have been identified by using *L. pneumophila* as a surrogate host (12, 20–23, 28). Many *C. burnetii* T4BSS substrates have features of eucaryotic proteins, such as ankyrin repeat domains, that may functionally mimic or antagonize the activity of host proteins (12, 21–23). However, only AnkG has a defined function in inhibiting apoptosis via binding of the proapoptotic protein gC1qR (p32) (12).

The historic obligate intracellular nature of *C. burnetii* has severely impeded the development of genetic tools to study type IVB secretion and other putative virulence factors. Fortunately, significant advances in the genetic manipulation of *C. burnetii* have recently been made (24). Using host cell-based propagation, Beare et al. (25) genetically transformed *C. burnetii* to chloramphenicol resistance and mCherry red fluorescent protein expression using the *mariner*-based *Himar1* transposon (Tn). Limitations of the procedure include the 2 to 3 months required for the isolation of individual transposon mutants and a technically challenging micro-manipulation cloning method. These problems were solved with the development of an axenic (host cell-free) method of *C. burnetii* propagation (26, 27). Under microaerobic conditions, a medium called acidified citrate cysteine medium 2 (ACCM-2) supports approximately 4 logs (\log_{10}) of *C. burnetii* growth in liquid medium and the formation of small (~0.5 mm in diameter) colonies in solid medium (26). A completely axenic *C. burnetii* genetic transformation system is now available that allows the isolation of genetic transformants in about 2.5 weeks. ACCM-2 recently facilitated the development of RSF1010 *ori*-based shuttle vectors that autonomously replicate in *C. burnetii* (22, 28). These shuttle vectors were used to confirm cytosolic delivery by *C. burnetii* of 15 Dot/Icm substrates originally identified utilizing the heterologous *L. pneumophila* system (20, 22, 28).

Axenic growth also allows the isolation of *C. burnetii* mutants incapable of growth in macrophages. Screening of colonies composed of clonal *C. burnetii* *Himar1* transformants revealed a clone with a transposon insertion in *icmD* (*dotP*). In *L. pneumophila*, IcmD is an inner membrane component of the Dot/Icm T4BSS (29) that is required for productive host cell infection (30). In the present study, we utilized the *C. burnetii* *icmD::Tn* mutant, along with new systems for genetic complementation and inducible gene expression, to probe the requirements of Dot/Icm function in *C. burnetii* parasitism of human macrophages.

RESULTS

A genetic transformant of *C. burnetii* contains a *Himar1* transposon insertion in *icmD*. We recently published an axenic protocol for the genetic transformation and clonal isolation of *C. burnetii* using ACCM-2 (26). An unpublished component of this study confirmed the clonality of randomly picked ACCM-2 colonies by mapping the transposon insertion sites of *C. burnetii* Nine Mile phase II (NMII) transformed with *Himar1*. One clone contained a transposon insertion at base 75 of the 414-bp *icmD* gene (Fig. 1A). Southern blotting confirmed a single transposon insertion in this transformant and, as predicted by the genome sequence, disruption of the *icmD*-containing 1.62-kb HindIII fragment (Fig. 1B). IcmD-specific antiserum did not detect the IcmD protein (14.3 kDa) in lysates of the *icmD::Tn* mutant by immunoblotting (Fig. 1C).

***icmD* is dispensable for uptake by macrophages but not productive infection.** To ascertain whether IcmD is required for the productive infection of macrophages, one-step growth curves of *C. burnetii* in THP-1 macrophages were determined and immunofluorescence microscopy was performed. The axenic replication of NMII and that of the *icmD::Tn* mutant were indistinguishable, with both strains attaining roughly 10,000-fold increases in genome equivalents (GE) over 6 days of incubation (Fig. 2A). Conversely, the *icmD::Tn* mutant showed an only 7.9-fold increase in GE at 9 days postinfection (p.i.) of THP-1 macrophages, whereas NMII GE increased by over 10,000-fold. At 6 days p.i., the *icmD::Tn* mutant did not form the single large, perinuclear localized PV typical of *C. burnetii*. Instead, the organism was generally lodged in tight-fitting vacuoles randomly dispersed through-

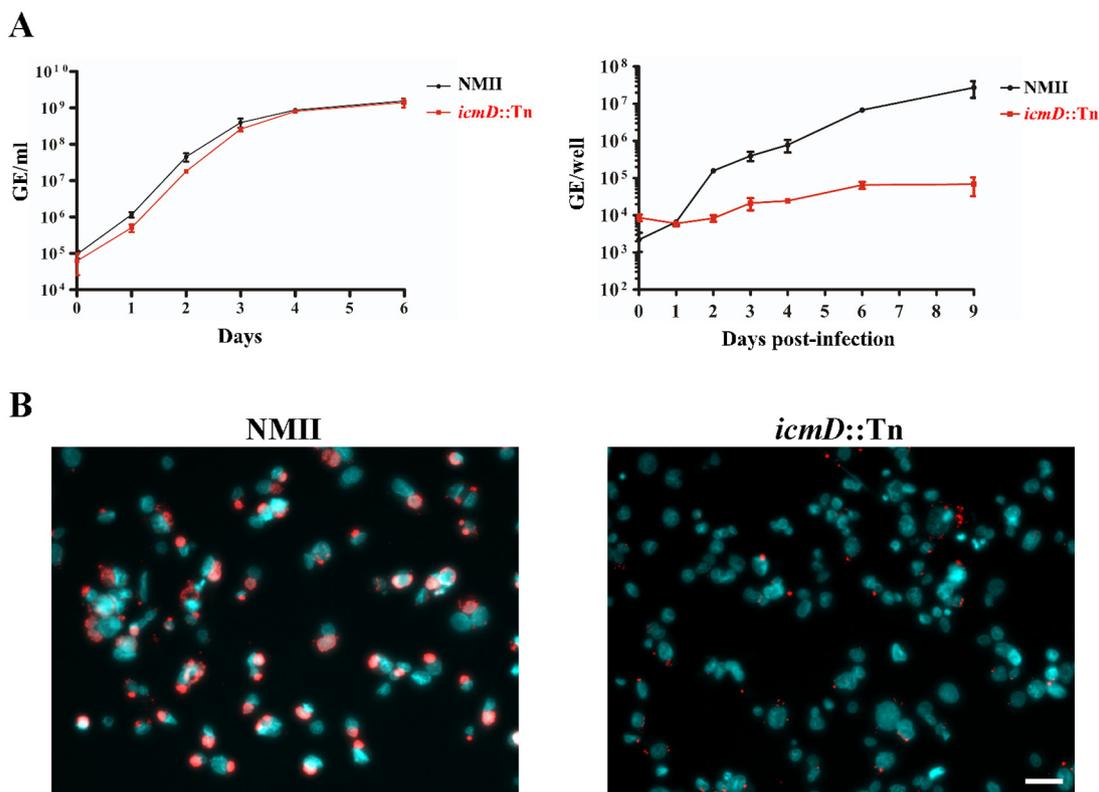


FIG 2 *icmD* is required for intracellular growth of *C. burnetii*. (A) One-step growth curves of NMII and the *icmD*::Tn mutant grown in ACCM-2 (left panel) or THP-1 macrophages (right panel). Growth was measured by enumerating GE. ACCM-2 results are expressed as the means of three biological replicates from one experiment and are representative of two independent experiments. Error bars indicate the standard deviations of the means. THP-1 macrophage results are expressed as the means of two biological replicates from one experiment and are representative of three independent experiments. Error bars indicate the standard deviations of the means. (B) Fluorescence micrographs of THP-1 macrophages infected for 6 days with NMII or the *icmD*::Tn mutant. *C. burnetii* (red) is stained by indirect immunofluorescence, and DNA (blue) is stained with DAPI. Cells contained multiplying NMII harbored within 1 or 2 large and spacious PVs per cell, while the *icmD*::Tn mutant was typically observed as a single organism in dispersed, tight-fitting PVs. Bar, 20 μ m.

out the cytoplasm (Fig. 2B). The growth deficiency of the *icmD* mutant was not due to defective uptake by THP-1 macrophages, as entry assays showed cells with equal numbers, i.e., 1.06 ± 0.097 and 1.18 ± 0.107 , of internalized NMII and *icmD*::Tn mutant bacteria, respectively.

icmD is required for cytosolic delivery of T4BSS substrates.

To confirm that the deficiency in intracellular replication of the *icmD*::Tn mutant correlated with defective cytosolic delivery of T4BSS substrates, the CyaA translocation assay was conducted using THP-1 macrophages infected with *C. burnetii* transformants expressing CyaA fusions to *C. burnetii* proteins CpeD and CpeE. Both proteins have been shown to be secreted into the host cytosol by *C. burnetii* using CyaA and β -lactamase translocation assays and by *L. pneumophila* in a DotA-dependent fashion using the CyaA assay (22). NMII and the *icmD*::Tn mutant were transformed with Tn7 constructs encoding CyaA alone or a CyaA fusion protein under the control of the constitutive CBU1169 promoter (22, 24). Tn7 was chosen over RSF1010 *ori* plasmids for transformations to circumvent potential gene dosage effects associated with plasmid copy number (~ 3 to 6) (24). We have shown that, as in other Gram-negative bacteria, Tn7 is inserted as a single copy into the *C. burnetii* chromosome in an intergenic region downstream of *glmS* (CBU1787), which encodes glucosamine-6-phosphate synthetase (24, 31).

CyaA translocation assays were conducted at 2 days p.i. CpeD

and CpeE fusion proteins were secreted by NMII as indicated by a greater-than-100-fold increase in cyclic AMP (cAMP) levels relative to cells harboring organisms expressing CyaA alone (Fig. 3). Conversely, secretion by the *icmD*::Tn mutant was not detected. Negative secretion was not due to a lack of CyaA fusion protein, as immunoblotting revealed the production of equal amounts of fusion protein by NMII and the *icmD*::Tn mutant (Fig. 3). Thus, *IcmD* is required for the secretion of type IVB effectors into the host cell cytosol.

***icmD* is required for inhibition of apoptosis.** *C. burnetii* protein synthesis is required to protect infected cells against apoptotic stimuli (13–15). Moreover, the *C. burnetii* T4BSS substrate AnkG has defined antiapoptotic activity (12). To determine if defective type IVB secretion by the *icmD*::Tn mutant correlated with reduced protection from apoptotic cell death, THP-1 macrophages infected for 2 days were treated for 4 h with staurosporine, an intrinsic apoptosis inducer. Following treatment, cells were scored for the presence of nuclear cleaved poly(ADP-ribose) polymerase (PARP), a marker of the terminal stages of apoptosis (32). Cultures infected with the *icmD*::Tn mutant had approximately the same percentage ($\sim 18.5\%$) of cleaved PARP-positive nuclei as uninfected cell cultures (Fig. 4). This percentage was significantly higher than that in cultures infected with NMII ($\sim 3.7\%$), thereby confirming that *C. burnetii* type IVB secretion is required to block apoptosis.

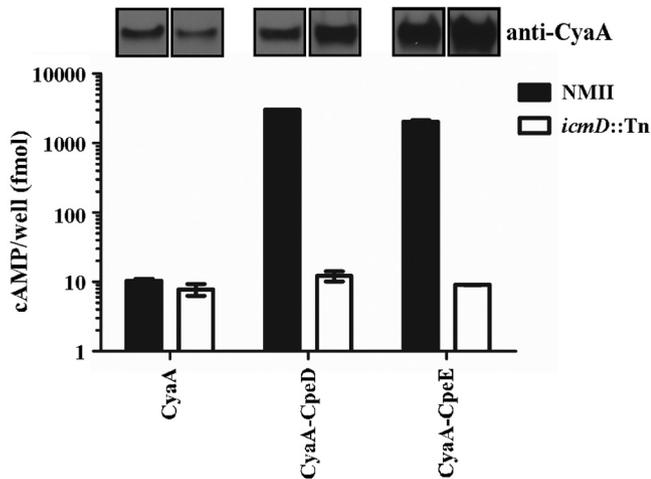


FIG 3 *icmD* is required for cytosolic delivery of T4BSS substrates. Cytosolic levels of cAMP following infection of THP-1 macrophages for 2 days with NMII or the *icmD::Tn* mutant expressing CyaA alone or CyaA fusions to the previously defined Dot/Icm substrates CpeD and CpeE. Elevated levels of cAMP, indicating secretion, were observed only with NMII expressing CyaA-CpeD or -CpeE fusion protein. Results shown are from one experiment conducted in duplicate and are representative of two independent experiments. Error bars indicate the standard errors of the means. Immunoblotting signals of CyaA and CyaA-effector fusion proteins, depicted above respective histogram bars, show comparable levels of protein expression by NMII and the *icmD::Tn* mutant after 6 days of growth in ACCM-2. Blots were probed with anti-CyaA antibody.

Coinfection with NMII or *L. amazonensis* rescues intracellular growth of the *icmD::Tn* mutant. Alone, the *icmD::Tn* mutant does not productively infect cultured macrophages. We were curious about whether growth could be rescued upon trafficking of the mutant to PVs harboring isogenic NMII, where Dot/Icm T4BSS functions would be provided in *trans*. THP-1 macrophages were coinfecting with NMII and the *icmD::Tn* mutant. Immunofluorescence microscopy revealed PVs filled with roughly equal numbers of each strain at 6 days p.i. (Fig. 5), suggesting that the *icmD::Tn* mutant replicates when coinhabiting a vacuole generated by NMII. GE measurements conducted at 6 days p.i. con-

firmed the enhanced replication of the *icmD::Tn* mutant during coinfection, with the mutant yielding 109.2 (± 4.7)-fold more GE than bacteria in singly infected cells ($P < 0.0001$).

The PV of *C. burnetii* and that of the protozoan parasite *L. amazonensis* are superficially similar in being fusogenic, moderately acidic (pH 5), and decorated with lysosomal markers (33). Moreover, in Chinese hamster ovary cells, *L. amazonensis* traffics to preexisting *C. burnetii* PVs, where it replicates (34). To establish whether the *icmD::Tn* mutant could traffic to and replicate within the foreign *L. amazonensis* PV, Vero cells infected with *L. amazonensis* for 1 day were superinfected with the *icmD::Tn* mutant. The infection was then allowed to proceed for 4 more days. This coinfection protocol was employed because, in our experiments, it optimized the generation of coinhabited PVs. These vacuoles were still found in less than 10% of the cells, which reflected the low infection efficiency of *L. amazonensis*. Nonetheless, the *icmD::Tn* mutant trafficked to *L. amazonensis* PVs and, without exception, replicated in this compartment, as indicated by vacuoles containing bacteria well in excess of the initial multiplicity of infection (MOI) of 1 (Fig. 5). Enumeration of the *icmD::Tn* mutant GE was not conducted due to the low number of coinhabited PVs within cell cultures. Nonetheless, these data indicate the *icmD::Tn* mutant is capable of intracellular growth in the total absence of Dot/Icm T4BSS effector functions if harbored in a suitable acidic compartment.

Complementation of the *icmD::Tn* mutant requires expression of *icmD*, *icmJ*, and *icmB*. The *C. burnetii* *icmD* gene is the first gene in a predicted operon including *icmJ* and *icmB* (35). Therefore, the transposon insertion in the *icmD::Tn* mutant could have a polar effect that requires complementation of the mutant with *icmD*, *-J*, and *-B*. Consequently, complementation studies were conducted using Tn7 plasmid constructs encoding *icmD* (pMiniTn7-CAT::*icmD^P-icmD*), *icmDJ* (pMiniTn7-CAT::*icmD^P-icmDJ*), and *icmDJB* (pMiniTn7-CAT::*icmD^P-icmDJB*) (see Table S1 and Fig. S1 in the supplemental material). These constructs contain 191 bp upstream of *icmD* predicted to encode the endogenous *icmD* promoter.

Functional complementation, as scored by significant GE increases at 6 days p.i. and the production of large PVs harboring

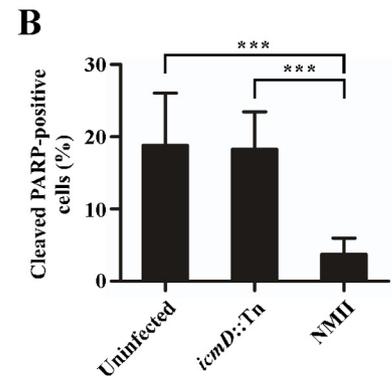
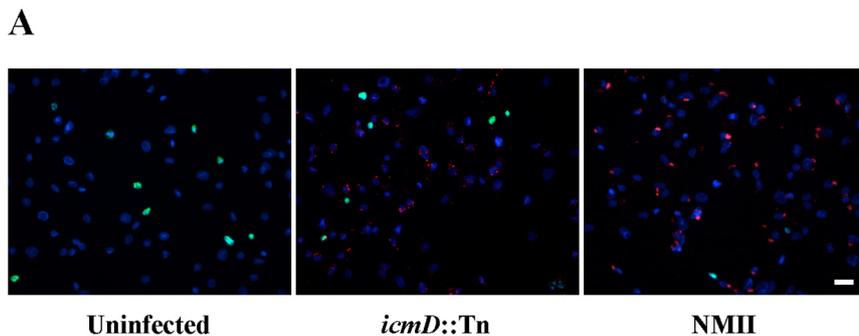


FIG 4 *icmD* is required for inhibition of apoptosis. THP-1 macrophages were infected with NMII or the *icmD::Tn* mutant for 2 days and then treated with staurosporine for 4 h to induce apoptosis. Uninfected cells were used as a control. (A) Detection of cleaved PARP-positive nuclei. *C. burnetii* (red) and cleaved PARP (green) were labeled by indirect immunofluorescence. DNA (blue) was stained with DAPI. Bar, 20 μ m. (B) Enumeration of cleaved, PARP-positive (apoptotic) nuclei. The results shown are from one experiment conducted in triplicate and are representative of two independent experiments. A total of 750 cells were counted for each condition. Error bars indicate the standard deviations of the means, and asterisks indicate a statistically significant difference ($P < 0.0005$) from cells infected with NMII.

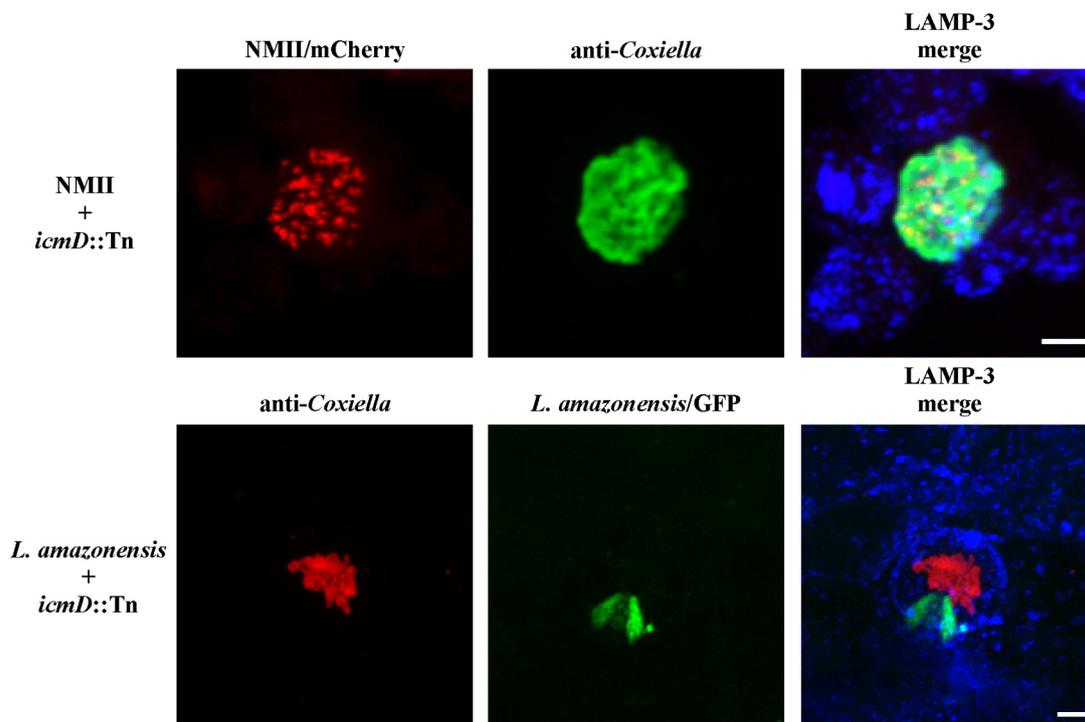


FIG 5 Coinfection with NMII or *L. amazonensis* rescues intracellular growth of the *icmD::Tn* mutant. (Top) THP-1 macrophages were coinfecting for 6 days with NMII expressing mCherry red fluorescent protein (24) and the *icmD::Tn* mutant. Both *C. burnetii* strains (green) and LAMP-3 (blue) were stained by indirect immunofluorescence. Confocal fluorescence micrographs show coinhabited PVs with replicating NMII (yellow due to red and green overlay) and *icmD::Tn* mutant bacteria (green only). Bar, 5 μm. (Bottom) Vero cells were infected for 1 day with *L. amazonensis* promastigotes expressing GFP and then superinfected for 4 days with the *icmD::Tn* mutant. The *icmD::Tn* mutant (red) and LAMP-3 (blue) were stained by indirect immunofluorescence. Confocal fluorescence micrographs showed coinhabited PVs with replicating *L. amazonensis* and *icmD::Tn* mutant bacteria. Bar, 5 μm.

replicating bacteria, was observed only with the *icmD::Tn* mutant transformed with Tn7::*icmDJB* (Fig. 6A and B). The GE yield of the *icmD::Tn* mutant transformed with Tn7::*icmD* or Tn7::*icmDJ* did not surpass that of the untransformed mutant. QuantiGene transcript analysis of *C. burnetii* cultivated in ACCM-2 for 4 days revealed negligible levels of *icmD*, *-J*, and *-B* transcripts in the *icmD::Tn* mutant that were restored to wild-type levels following single-copy, in *cis* complementation with Tn7::*icmDJB* (Fig. 6C). Conversely, CBU1169, encoding the small heat shock protein Hsp20, was equally expressed by NMII, the *icmD::Tn* mutant, and the complemented *icmD::Tn* mutant (Fig. 6C). Immunoblotting confirmed the production of IcmD by the complemented mutant (Fig. 6D). Collectively, our complementation results support an operon structure for *icmD*, *-J*, and *-B* and confirm that deficient intracellular replication of the *icmD::Tn* mutant is due to inactivation of the Dot/Icm T4BSS.

Induction of *icmDJB* expression after infection rescues growth of the *icmD::Tn* mutant. Successful complementation of the *icmD::Tn* mutant allowed examination of the temporal requirements of type IVB secretion in productive infection by *C. burnetii*. These experiments required the development of a system for tightly regulated expression of the *icmDJB* gene cluster. This was achieved by using a Tn7 plasmid construct containing *icmDJB* under the control of an anhydrotetracycline (aTc)-inducible promoter (pMiniTn7-CAT::TetRA-*icmDJB*) (see Fig. S1 in the supplemental material). By immunoblotting, IcmD synthesis by the *icmD::Tn* mutant transformed with Tn7::TetRA-*icmDJB* was associated only with induced cultures (Fig. 7A).

THP-1 macrophages were infected with the *icmD::Tn* mutant transformed with Tn7::TetRA-*icmDJB*, where aTc was added to the culture medium at 0 h or 1 day p.i. These infection conditions were termed concurrent induction (CI) and delayed induction (DI), respectively, and aTc was present in culture medium throughout the infection time course. Infections were also conducted without aTc (no induction, NI). At 6 days postinduction, the CI and DI conditions yielded 50.7- and 9.1-fold increases in transformant GE, respectively (Fig. 7B), and production of large and spacious, LAMP-3-positive PV (Fig. 7C and data not shown). These GE increases were significantly greater than the 4.2-fold GE increase of the transformant under NI conditions, where organisms were found in tight-fitting, LAMP-3-positive phagosomes (Fig. 7B and C).

The CyaA translocation assay confirmed that the enhanced replication associated with *icmDJB* induction correlated with a renewed ability to secrete T4BSS substrates. The *icmD::Tn* mutant was transformed with a Tn7 construct encoding aTc-inducible *icmJBD* and a CyaA fusion to CpeD expressed from the constitutive CBU1169 promoter (pMiniTn7-CAT::TetRA-*icmDJB*-1169P-*cyaA-cpeD*) (see Fig. S1 in the supplemental material). Assays were conducted using lysates of THP-1 macrophages infected for 2 days with this transformant, with or without aTc induction at 1 day p.i. Cytosolic delivery of CyaA-CpeD by the transformed *icmD::Tn* mutant occurred only under inducing conditions (Fig. 7D).

Collectively, these data indicate that *C. burnetii* can initiate significant replication in host cells even when Dot/Icm T4BSS host

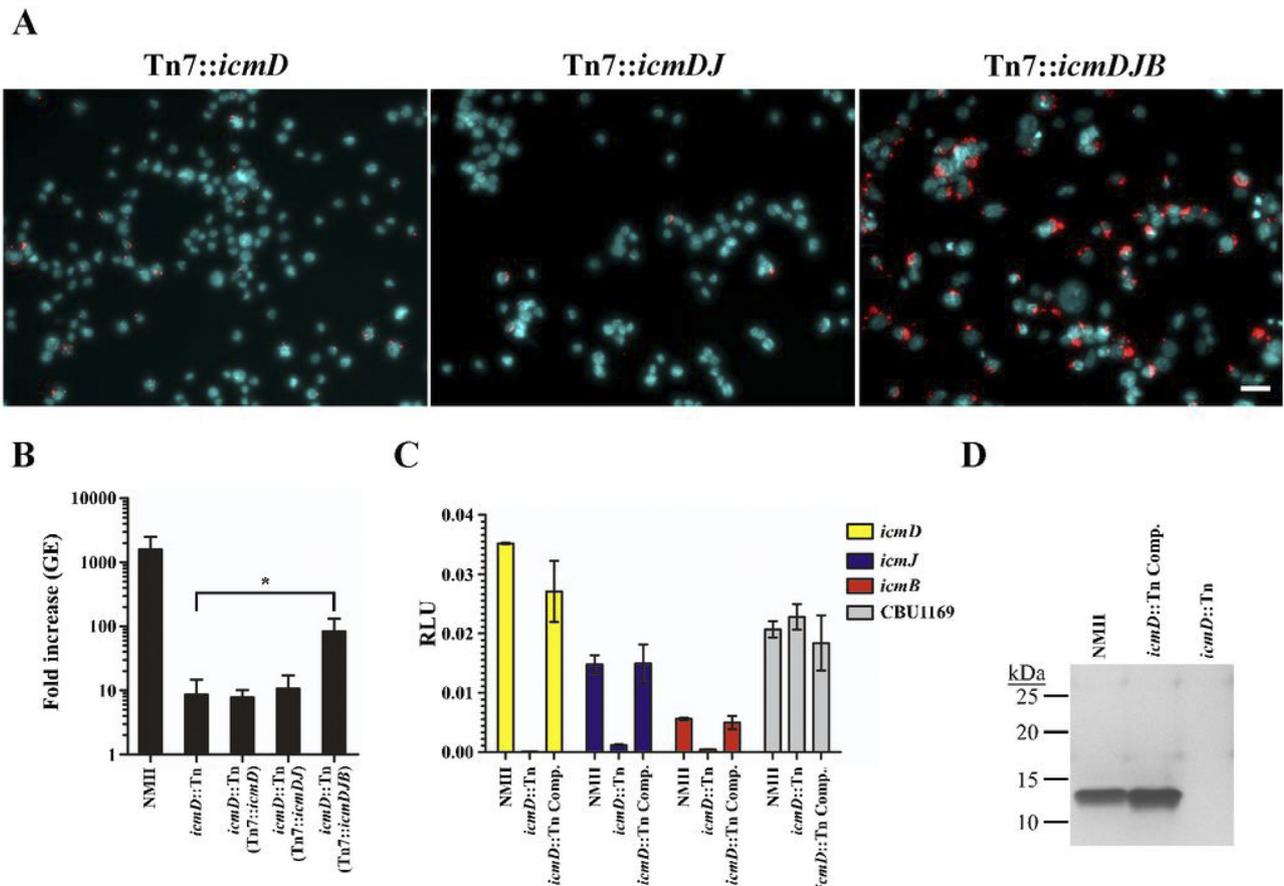


FIG 6 Intracellular growth of the *icmD*::Tn mutant requires the expression of *icmD*, *icmJ*, and *icmB*. (A) THP-1 macrophages infected for 6 days with the *icmD*::Tn mutant transformed with a Tn7 construct containing *icmD*, *icmDJ*, or *icmDJB* under the control of a native *C. burnetii* promoter. *C. burnetii* (red) was stained by indirect immunofluorescence, and DNA (blue) was stained with DAPI. Fluorescence micrographs show large PVs harboring replicating *C. burnetii* only within cells infected with the *icmD*::Tn mutant transformed with Tn7::*icmDJB*. Bar, 20 μm. (B) Increases in GE of NMII or the *icmD*::Tn mutant transformed with Tn7::*icmD*, Tn7::*icmDJ*, or Tn7::*icmDJB* after 6 days of growth in THP-1 macrophages. The asterisk indicates a statistically significant difference ($P < 0.05$) between the *icmD*::Tn mutant and the mutant transformed with Tn7::*icmDJB*. (C) *icmD*, *icmJ*, *icmB*, and CBU1169 transcript levels in NMII, the *icmD* mutant, and the *icmD* mutant complemented with Tn7::*icmDJB* (*icmD*::Tn comp) after 4 days of growth in ACCM-2. Expression is shown as relative light units (RLU). (D) Immunoblotting of lysates of *C. burnetii* grown in ACCM-2 for 6 days showing production of IcmD by the complemented mutant and NMII. The experiments depicted in panels B and C were performed in triplicate, and error bars indicate the standard errors of the means.

cell-modulating functions are deployed well after pathogen uptake. Moreover, these data show that the mutant retains viability in a nonpermissive lysosome-like vacuole.

DISCUSSION

Since the release of the Nine Mile reference strain genome sequence, the predicted Dot/Icm T4BSS of *C. burnetii* has been considered essential for pathogen colonization of macrophage host cells without supporting experimental evidence (36–38). In this study, we present data proving that Dot/Icm function is required for productive macrophage infection. Our *icmD*::Tn mutant has no defect in axenic growth or internalization by THP-1 macrophages. However, intracellularly, the mutant has severe growth defects, fails to generate a large PV, and does not protect cells from apoptosis. These defects correlate with failed cytosolic translocation of the Dot/Icm T4BSS substrates CpeD and CpeE. Our results are consistent with findings recently published by Carey et al. (20) on epithelial cell interactions of an NMII mutant with *icmL* inactivated by *Himar1*. The *icmL*::Tn mutant shows no defect in axenic

growth or uptake by HeLa cells but does not replicate in either HeLa or Vero cells. Furthermore, this mutant fails to translocate three *C. burnetii* Dot/Icm T4BSS substrates (CBU0077, CBU0635, and CBU1524). Thus, two independent studies have confirmed the critical importance of the Dot/Icm T4BSS in *C. burnetii* infection of disparate host cell types.

In the present study, we used a newly established *C. burnetii* transformation system based on Tn7 to complement the *icmD*::Tn mutant. A Tn7 insertion in the *glmS*-CBU1788 intergenic region has no demonstrable effect on the axenic and *in vivo* growth of *C. burnetii* while having the added benefit of allowing single-copy, *in cis* complementation (24, 31). Complementation required functional expression of *icmD*, *-J*, and *-B*. This result is consistent with an operon prediction by the Database of Prokaryotic Operons (35) and the observation that transposon inactivation of *icmD* results in dramatically reduced transcription of downstream *icmJ* and *-B*. The transcriptional arrangement of *C. burnetii* *icmD* differs from that of *L. pneumophila* where *icmD* is predicted to lie at the end of an eight-gene operon including *lphA* and *icmMLKEGCD* (30, 35).

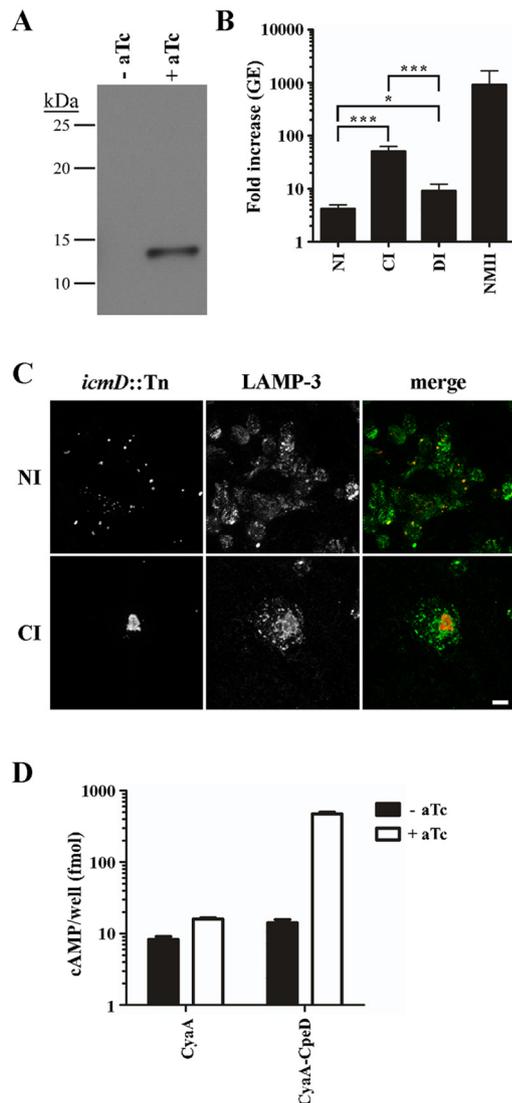


FIG 7 Induction of *icmDJB* expression after infection rescues the growth of the *icmD::Tn* mutant. (A) aTc-inducible expression of *icmD*. The *icmD::Tn* mutant transformed with a Tn7 construct containing aTc-inducible *icmDJB* was cultivated in ACCM-2 for 3 days and then induced with aTc for 1 day. Immunoblotting showed aTc-induced synthesis of IcmD. (B and C) aTc induction of *icmDJB* concurrent with or after infection results in PV production and significant replication by the *icmD::Tn* mutant at 6 days postinduction. THP-1 macrophages were infected with the *icmD::Tn* mutant transformed with Tn7::TetRA-*icmDJB*, where aTc was added to the culture medium at 0 h (CI) or 1 day (DI) p.i. Transformant infections were also conducted without aTc (NI) and with NMII. (B) One asterisk ($P < 0.05$) and three ($P < 0.0005$) asterisks indicate significant differences in GE. The experiment was performed three times in duplicate, and error bars indicate the standard deviations of the means. (C) The *icmD::Tn* mutant grown under CI, but not NI, conditions forms typical large, LAMP-3-positive PVs at 6 days p.i. *C. burnetii* and LAMP-3 were stained by indirect immunofluorescence and appear red and green, respectively, in the merged image. Bar, 10 μ m. (D) Induced expression of *icmDJB* restores T4BSS function by the *icmD::Tn* mutant. THP-1 macrophages were infected with the *icmD::Tn* mutant transformed with a Tn7 construct encoding aTc-inducible *icmDJB* and a CyaA fusion to CpeD expressed from the constitutive CBU1169 promoter. *C. burnetii* expressing CyaA alone was used as a control. For induction of *icmDJB* expression, aTc was added at 1 day p.i. CyaA assays were conducted at 2 days p.i. Elevated levels of cAMP, indicating secretion, were observed only with the complemented mutant expressing CyaA-CpeD under inducing conditions. The results shown are from one experiment conducted in duplicate and are representative of two independent experiments. Error bars indicate the standard errors of the means.

The *icmD::Tn* mutant resides in a nonfusogenic and LAMP-3-positive phagosome that does not expand to form the large and spacious PV that supports robust *C. burnetii* replication (5, 10). However, at 6 days p.i., we consistently observed *icmD::Tn* mutant GE increases of approximately 5-fold that appear to result from bacterial division within the first few days of infection. We speculate that the mutant is capable of a few rounds of replication in the acidic but nutritionally deficient phagolysosome, with replication ceasing after the depletion of endogenous and/or exogenous nutrient sources. Relative to the uninduced *icmD::Tn* mutant, induction of *icmDJB* expression with aTc at 1 day p.i. results in a significant increase in GE, production of a typical PV, and a renewed ability to secrete Dot/Icm T4BSS substrates. However, the GE yields are significantly less than that of the *icmD::Tn* mutant induced at 0 h p.i., suggesting that the mutant loses some viability over time. This behavior may reflect the acid-activated metabolism of *C. burnetii* where loss of adenylate energy charge and infectivity is observed when organisms are incubated in an acidic buffer deficient in oxidizable energy sources (6, 39). Consequently, the *icmD::Tn* mutant may gradually lose viability while maintaining a detectable genome because the nutrient pool is insufficient to maintain ATP levels. The persistence of viable-but-nonreplicating *C. burnetii* in a phagolysosome was previously observed in NMII-infected Vero cells treated with chloramphenicol (11). The lower GE yield of the *icmD::Tn* mutant induced at 0 h p.i. relative to NMII may also suggest suboptimal aTc induction of the *icmDJB* operon.

The *icmD::Tn* mutant is capable of intracellular replication if sequestered in a PV generated by isogenic NMII. A *dotA* mutant of *L. pneumophila* behaves similarly in macrophages, with a strict requirement for synchronous coinfection with wild-type *L. pneumophila* (40). A more intriguing behavior is replication of the *icmD::Tn* mutant in the phagolysosome-like PV of *L. amazonensis*, a situation where there is a complete absence of Dot/Icm T4BSS-modulating functions. To support the growth of the mutant, the biochemical properties of the *Leishmania* vacuole must closely mimic those of the *C. burnetii* PV or growth-permissive ACCM-2. In axenic medium, amino acids are preferred carbon and energy sources of *C. burnetii* and growth declines precipitously when the oxygen level surpasses 7.5% (27). Thus, and as suggested for the *C. burnetii* PV (41), the *L. amazonensis*-containing vacuole, in addition to the low pH required for *C. burnetii* metabolic activation, likely has ample supplies of amino acids and a low oxygen content. Indeed, due to multiple amino acid auxotrophies, *Leishmania* spp. appear specifically adapted to growth in a hexose-poor but amino acid-rich modified phagolysosome of macrophages (42). As invoked for the *C. burnetii* PV (9), fusion between the *L. amazonensis* PV and autophagosomes is predicted to provide important nutrients, including amino acids (8, 43).

There are striking differences between *C. burnetii* and *L. pneumophila* in their temporal requirements for Dot/Icm function, a distinction that reflects their unique intracellular replication niches. To promote the development of an endoplasmic reticulum-derived vacuole that supports replication, *L. pneumophila* must express DotA prior to infection (44). Failure to do so results in rapid fusion (within 15 min) of the nascent phagosome with lysosomes and a lack of pathogen replication. These fates are observed for most *L. pneumophila dot/icm* null mutants (44, 45). Induction of DotA expression during bacterial uptake does not rescue pathogen replication. Conversely, both NMII and the *icm-*

Dot:Tn mutant traffic by default through the canonical endolysosomal pathway. The *icmD*:Tn mutant retains viability in a nonfusogenic phagolysosome and can therefore deploy T4BSS effectors that promote PV development and pathogen growth at 1 day p.i. The *Dot/Icm* system also participates in the uptake of *L. pneumophila*, with wild-type bacteria phagocytosed 10 to 20 times more efficiently by human macrophages than *dot/icm* mutants (46). Conversely, NMII and the *icmD*:Tn mutant are equally internalized, a result consistent with a previous report showing similar rates of uptake of viable and inactivated *C. burnetii* by murine L929 cells (47). Because *C. burnetii* is metabolically quiescent at neutral pH (6), secretion of effectors that induce uptake would not be expected.

In summary, this study confirms that successful macrophage parasitism by *C. burnetii* requires subversion of host cell functions by a repertoire of secreted *Dot/Icm* effector proteins. The generation of *C. burnetii dot/icm* mutants allows careful dissection of the specific cellular processes mediated by type IVB secretion, such as apoptotic signaling and PV biogenesis. This work also illustrates the growing amenability of *C. burnetii* to genetic manipulation.

MATERIALS AND METHODS

***C. burnetii*, *L. amazonensis*, *Escherichia coli*, and mammalian cells.** *C. burnetii* NMII (clone 4, RSA439) was used in this study. NMII was axenically cultivated in ACCM-2 as previously described (26). *L. amazonensis* IFLA/BR/67PH8/GFP was cultured as described by Wilson et al. (48). *E. coli* strains TOP10, PIR1, and BL21-AI (Invitrogen, Carlsbad, CA) were used for recombinant DNA procedures. THP-1 cells, a human acute monocytic leukemia cell line (TIB-202; ATCC), and African green monkey kidney (Vero) cells (CCL-81; ATCC) were maintained in RPMI 1640 medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) at 37°C and 5% CO₂.

Cell culture infection and aTc induction. With the exception of *C. burnetii*-*L. amazonensis* coinfections, all experiments utilized phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells, which accurately mimic the properties of human primary macrophages (49). To induce differentiation into macrophage-like cells, THP-1 were cells treated with 200 nM PMA (EMD Biosciences, San Diego, CA) for 2 days. Cells were washed twice with phosphate-buffered saline (PBS; 1 mM KH₂PO₄, 155 mM NaCl, 3 mM Na₂HPO₄, pH 7.4) to remove PMA prior to infection. For entry assays, cells were incubated for an additional 3 days in fresh RPMI medium following PMA treatment. This procedure induces a flattened cell morphology (49) that assists in the microscopic enumeration of internalized *C. burnetii* bacteria. Coinfection and growth assays used cells infected at an MOI of 1; cleaved PARP assays used cells infected at an MOI of 25; and CyaA, QuantiGene, immunoblotting, and entry assays used cells infected at an MOI of 200. *L. amazonensis* infection of Vero cells was conducted with stationary-phase promastigotes at an MOI of 50. With the exception of apoptosis assays, all inocula were removed after a 2-h incubation, cells were washed twice, and then fresh medium was added to infected cell cultures. This time point was considered 0 h p.i. With the exception of entry assays, all experiments used 24-well tissue culture plates. For entry assays, ibiTreat tissue culture-treated channeled μ -Slides V10.4 (ibidi, LLC, Verona, WI) were employed. Evaluation of *C. burnetii* replication in ACCM-2 or THP-1 macrophages was conducted by quantitative PCR (Q-PCR) of GE using a primer-probe set specific to the *dotA* gene as previously described (5, 27). Replication of mutant *C. burnetii* during coinfection with wild-type bacteria was specifically quantified by Q-PCR using a primer-probe set specific to the kanamycin resistance gene (*nptII*). Where indicated, aTc (Sigma-Aldrich, St Louis, MO) was added to *C. burnetii* ACCM-2 cultures and infected THP-1 macrophage cultures at final concentrations of 200 and 400 ng/ml, respectively, for induction of *C. burnetii* transformant gene expression. Where indicated, staurosporine (EMD Biosciences) was

added to cell cultures at a final concentration of 500 nM to induce apoptosis.

Plasmid construction. The plasmids used in this study are listed in Table S1 in the supplemental material. Details of plasmid construction are given in Table S2 in the supplemental material. DNA sequences were amplified by PCR using Accuprime *pfv* polymerase (Invitrogen) and gene-specific primers (Integrated DNA Technologies, Coralville, IA).

Transformation with *Himar1* and Tn7. *C. burnetii* cultured in ACCM-2 was transformed by electroporation as previously described (26). For transformation with *Himar1*, *C. burnetii* was coelectroporated with two suicide plasmids that individually code for the *Himar1* transposon (pITR-Kan) and the *Himar1* transposase (pUC19::1169P-*Himar1C9*) (see Fig. S2 in the supplemental material). For transformation with miniTn7, *C. burnetii* was coelectroporated with two suicide plasmids that individually code for the Tn7 transposase (pTnS2::1169P-*tsABCD*) and modified Tn7 (derivatives of pMiniTn7T-CAT) (24). Maps of the pMiniTn7T-CAT constructs used in this study are shown in Fig. S1 in the supplemental material. The genomic locations of *Himar1* insertions were determined using a modified version of a semirandom two-step PCR protocol described by Chun et al. (50). The Tn-specific primers used in the first- and second-round PCR amplifications were ColE1-3' out (5' A AGGGAGAAAGCGGACAGG 3') and ColE1-3' out-nested (5' CGCCT GGTATCTTTATAGTCCTGTC 3'), respectively. All of the *C. burnetii* transformants used in this study were cloned by colony picking.

PV and cleaved PARP staining. Methanol fixation and staining procedures for indirect immunofluorescence assays were conducted as previously described (11). Guinea pig anti-*C. burnetii* serum, rabbit anti-cleaved PARP serum (Cell Signaling Technology, Danvers, MA), and a mouse monoclonal antibody directed against LAMP-3 (CD63) (clone H5C6; BD Biosciences) were used as primary antibodies. Alexa Fluor 488 and 594 IgG (Invitrogen) were used as secondary antibodies. Coverslips were mounted using ProLong Gold containing 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) to visualize DNA. Epifluorescence microscopy images were acquired with a TE-2000 microscope equipped with a CoolSNAP HQ digital camera (Roper Scientific, Tucson, AZ) or a Ti-U microscope equipped with a DS-Qi1Mc camera (Nikon, Melville, NY). Confocal fluorescence microscopy was performed with a modified Perkin-Elmer UltraView spinning-disc confocal system connected to a Nikon Eclipse Ti-E inverted microscope. Confocal images (0.2- μ m sections) were acquired with a 63 \times oil immersion objective and a Photometrics Cascade II: 512 digital camera (Princeton Instruments, Trenton, NJ) using Metamorph software (Molecular Devices, Inc., Downingtown, PA). All images were processed similarly using ImageJ software (written by W. S. Rasband at the U.S. National Institutes of Health, Bethesda, MD, and available at <http://rsb.info.nih.gov/ij/>).

Entry assay. THP-1 macrophages were exposed to *C. burnetii* for 2 h, fixed with 2.5% paraformaldehyde for 15 min on ice, and then washed three times with PBS. Subsequent steps were done at room temperature. Cells were blocked with 1% bovine serum albumin (BSA) in PBS for 15 min and then incubated with rabbit anti-*C. burnetii* serum for 15 min. Cells were washed, permeabilized with 0.1% Triton X-100 in 1% BSA-PBS for 15 min, and then incubated with guinea pig anti-*C. burnetii* serum for 15 min. Cells were washed and then incubated with anti-rabbit Alexa Fluor 594 and anti-guinea pig Alexa Fluor 488 for 15 min. Cells were mounted in ProLong gold containing DAPI. Intracellular bacteria (green only) were enumerated by epifluorescence microscopy. The experiment was conducted three times in triplicate, and 100 host cells were counted per replicate.

QuantiGene transcript analysis. mRNA quantification was performed using the QuantiGene reagent system v.2.0 (Panomics, Santa Clara, CA) as previously described (22) and custom designed probes specific to *icmD*, *icmJ*, *icmB*, and CBU1169 (22). RNA was extracted from *C. burnetii* after 4 days of growth in ACCM-2.

IcmD antisera production. The *C. burnetii icmD* gene was cloned without its predicted 5' N-terminal signal anchor sequence-encoding re-

gion (bp 1 to 84) into the 6×His expression vector pEXP1 (Invitrogen) to generate pEXP1::*icmD*-SP (see Table S2 in the supplemental material). *E. coli* BL21/pEXP1::*icmD*-SP was induced for 4 h with 0.2% arabinose and 6×His-IcmD-SP purified by Ni²⁺ chromatography using a His-Bind buffer kit (EMD Biosciences) in the presence of 6 M urea. Urea was removed using an Amicon Ultra centrifugal filter (3-kDa cutoff; Millipore Corp., Billerica, MA). Purified 6×His-IcmD-SP (250 μg) in 25 mM Tris buffer (pH 7.2) with 0.05% Triton X-100 was mixed with the Sigma Adjuvant System and used to immunize a New Zealand White rabbit. Rabbit antiserum was generated according to protocol 2008-32.1, approved by the Rocky Mountain Laboratories Animal Care and Use Committee.

Immunoblotting. *C. burnetii* expression of CyaA fusion proteins or IcmD was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting. Membranes were incubated with rabbit anti-IcmD polyclonal antibody or a mouse monoclonal antibody directed against CyaA (clone 3D1; Santa Cruz Biotechnology, Santa Cruz, CA). Reacting proteins were detected using anti-rabbit (IcmD) or anti-mouse (CyaA) IgG secondary antibodies conjugated to horseradish peroxidase (Pierce, Rockford, IL) and chemiluminescence using ECL Pico reagent (Pierce).

CyaA translocation assay. CyaA translocation assays were performed as previously described, using the cAMP enzyme immunoassay (GE Healthcare, Piscataway, NJ) (23).

Statistical analysis. Statistical analyses were performed using the unpaired Student *t* test and Prism software (GraphPad Software, Inc., La Jolla, CA).

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00175-11/-/DCSupplemental>.

Figure S1, PDF file, 0.4 MB.

Figure S2, PDF file, 0.2 MB.

Table S1, PDF file, 0.1 MB.

Table S2, PDF file, 0.1 MB.

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